Plant Signaling & Behavior 4:9, 849-852; September 2009; © 2009 Landes Bioscience

The ER body, a new organelle in *Arabidopsis thaliana*, requires NAI2 for its formation and accumulates specific β-glucosidases

Kenji Yamada,[1,2] Atsushi J. Nagano,[3,†] Kimi Ogasawara,[1,2,‡] Ikuko Hara-Nishimura[3] and Mikio Nishimura[1,2,*]

[1]Department of Cell Biology; National Institute for Basic Biology; Okazaki, Aichi Japan; [2]School of Life Science; Graduate University for Advanced Studies (Sokendai); Okazaki, Aichi Japan; [3]Department of Botany; Graduate School of Science; Kyoto University; Sakyo, Kyoto Japan

Current addresses: [†]Photobiology and Photosynthesis Research Unit; National Institute of Agrobiological Sciences; Tsukuba, Ibaraki Japan; [‡]Research Institute for Electronic Science; Hokkaido University; Sapporo, Hokkaido Japan

Plants develop various ER-derived structures with specific functions. The ER body found in *Arabidopsis thaliana* is a spindle-shaped structure. ER bodies accumulate in epidermal cells in seedlings or are induced by wounding. The molecular mechanisms underlying the formation of the ER body remained obscure. We isolated an ER body-deficient mutant in Arabidopsis seedlings, which we termed *nai2*. The *NAI2* gene encodes a member of a unique protein family. NAI2 localizes to the ER body and the downregulation of *NAI2* elongates ER bodies and reduces their number. ER bodies specifically accumulate high levels of PYK10/BGLU23, which is a β-glucosidase that bears an ER retention signal. Additionally, in the *nai2* mutant, PYK10 protein is diffuse throughout the ER and the PYK10 protein level is reduced. These observations indicate that NAI2 is a key factor for the formation of ER bodies and for the accumulation of PYK10 in the ER bodies of Arabidopsis. We also found that *BGLU18*, which encodes another β-glucosidase with an ER retention signal, is induced at the site of wounding. Immunocytochemical analysis revealed that the BGLU18 protein is exclusively localized in ER bodies formed directly at the wounding site of cotyledons. These results suggest that BGLU18 is a component of the ER body in wounded leaves of Arabidopsis.

Key words: endoplasmic reticulum, ER body, b-glucosidase, *Arabidopsis thaliana*, seedling, wounding

Abbreviations: BGLU, β-glucosidase; ER, endoplasmic reticulum; GFP, green fluorescent protein; GLL, GDSL lipase-like protein; JAL, jacalin-related lectin; PBP1, PYK10 binding protein 1; RNAi, RNA interference; TSA1, TSK-associating protein 1

Submitted: 06/15/09

Accepted: 06/16/09

Previously published online: www.landesbioscience.com/journals/psb/article/9377

*Correspondence to:
Mikio Nishimura; Email: mikosome@nibb.ac.jp

Addendum to: Yamada K, Nagano AJ, Nishina M, Hara-Nishimura I, Nishimura M. NAI2 is an endoplasmic reticulum body component that enables ER body formation in *Arabidopsis thaliana*. Plant Cell 2008; 20:2529–40; PMID: 18780803; DOI: 10.1105/tpc.108.059345.
and
Ogasawara K, Yamada K, Christeller JT, Kondo M, Hatsugai N, Hara-Nishimura I, Nishimura M. Constitutive and inducible ER bodies of *Arabidopsis thaliana* accumulate distinct beta-glucosidases. Plant Cell Physiol 2009; 50:480–8; PMID: 19147648; DOI: 10.1093/pcp/pcp007.

Proteins that eventually enter the secretory pathway are synthesized on the rough ER where the ribosomes are attached. These newly synthesized proteins are then modified by disulfide bond formation and attachment of oligosaccharides, which take place in the ER lumen, before being transported to their destination via vesicle trafficking. Most transport vesicles moving from the ER are coat protein II vesicles of ~50 nm in diameter. Plants differ from animals in that they also produce different types of ER-derived vesicles involved in the accumulation of unique types of proteins.[1,2]

We have identified a distinct type of ER-derived structure as a new organelle in Arabidopsis, which we have designated the ER body.[3] The spindle-shaped, 5–10 μm long ER bodies were easily detected in Arabidopsis expressing ER-targeted GFP (**Fig. 1A**).[4] Electron microscopic analysis revealed that ER bodies have a fibrous structure and are surrounded by a single ribosome-bearing membrane (**Fig. 1B**).[4] ER bodies are distributed throughout the epidermis of cotyledons and hypocotyls in young seedlings, and subsequently disappear with plant growth.[5] In contrast, the majority of the root tissues constitutively accumulate ER bodies.[6] Interestingly, wounding or treatment with the wound hormone jasmonate induces the accumulation of ER bodies in adult leaves.[5] This suggests that the ER body is involved in pest/pathogen resistance in Arabidopsis.[7] Structures that are similar to the ER bodies of Arabidopsis

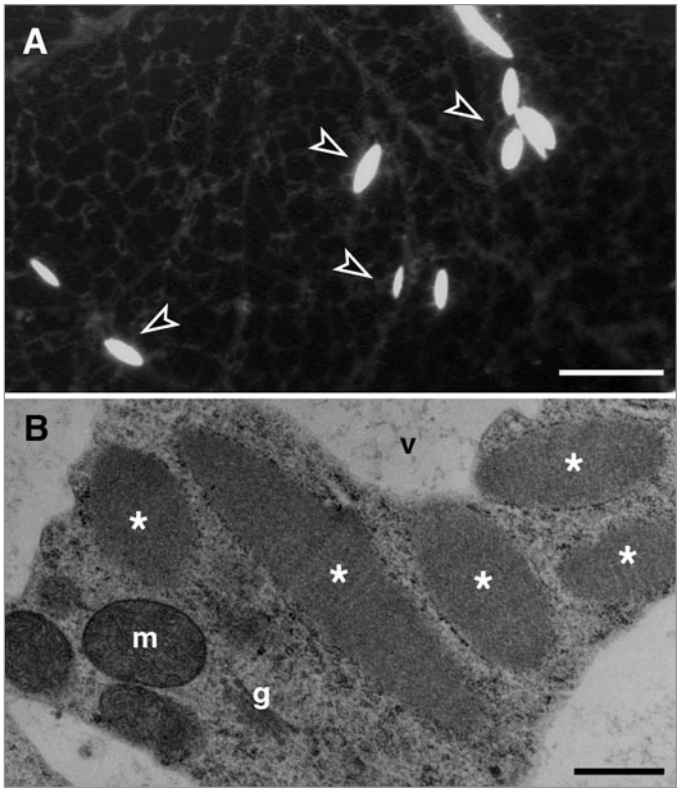

Figure 1. ER bodies in epidermal cells of Arabidopsis cotyledons. (A) Fluorescent image of transgenic plants that expressed ER-targeted GFP. Arrowheads indicate ER bodies. Scale bar = 10 μm. (B) Electron micrograph of ER bodies. Asterisks indicate the ER bodies. m, mitochondrion; g, Golgi body; v, vacuole. Scale bar = 0.5 μm. Reproduced from Hayashi et al.[4] with permission from Oxford University Press.

have also been reported in the cells of various organs of other Brassicales plants, which include *Arabis alpina*, *Brassica oleracea*, *Raphanus sativus*, *Capparis spinosa* and *Cleome spinosa*.[8-10]

The ER bodies in Arabidopsis seedlings accumulate PYK10/BGLU23/At3g09260 protein, which bears the ER retention signal KDEL.[6] PYK10 is the major component of the ER body.[6] As the electron microscopic analysis revealed that there are high-density materials in the ER body, it seems likely that PYK10 accumulates as a condensed (or aggregated) material in the ER body.

The Arabidopsis *nai1* mutant (nai means "nothing" in Japanese) lacks ER bodies. The *NAI1/At2g22770* gene encodes a basic helix-loop-helix-type transcription factor.[11] NAI1 regulates the expression of *PYK10*, *JAL22/At2g39310*, *JAL23/At2g39330*, *JAL31/At3g16430*, *JAL33/At3g16450*, *PBP1/JAL30/At3g16420*, *GLL23/At1g54010* and *GLL25/At1g54030*.[11-13] PBP1 localizes to the cytosol, while PYK10 localizes to the ER body.[11,13] Recently, we found that PYK10 forms a large complex with JALs and GLLs when cells were collapsed by wounding.[12] JALs and GLLs regulate the size of the PYK10 complex and may regulate its substrate specificity. As NAI1 deficiency causes the loss of ER bodies, NAI1 may regulate unknown factors that are responsible for the formation of the ER body.

NAI2 is an ER Body Component Necessary for ER Body Formation and for the Accumulation of PYK10

We searched for mutants that lack ER bodies from T-DNA insertion lines. The *nai2-1* mutant, which carries a single recessive mutation, lacks ER bodies in all parts of the seedling.[14] The phenotype of the *nai2-1* mutant is very similar to that of the *nai1* mutant. This suggests that *NAI1* regulates the expression of the *NAI2* gene and that the expression of *NAI2* is down-regulated in *nai1*. We analyzed microarray expression data of *nai1-1*,[12] and focused on the uncharacterized gene *At3g15950*. A subsequent genome analysis revealed that the *nai2-1* mutant lacks the *At3g15950* gene. The insertion of a genome fragment containing the *At3g15950* gene rescues the phenotype of the *nai2-1* mutant, which indicates that the *NAI2* gene is *At3g15950*.

The NAI2 protein comprises 772 amino acids and has a signal peptide at its N-terminus, which suggests that NAI2 is a secretory protein. The N-terminal half of NAI2 has a Glu-Phe-Glu (EFE) motif consisting of ten repeats of a sequence of ~40 amino acids.[15] The sequence of the C-terminal region of NAI2 is unique; we termed it the NAI2 domain. Two proteins in Arabidopsis are structurally related to NAI2, i.e., TSA1/At1g52410 and At3g15960.[15] No *NAI2* homologous genes could be found in animals, fungi or unicellular organisms; however, we identified *NAI2* homologous genes in *B. rapa*, *B. oleracea* and *B. napus*. These findings suggest that *NAI2* homologous genes are unique to Brassicales plants.

To determine the subcellular localization of the NAI2 protein, we raised antibodies against a NAI2 polypeptide and performed an immunofluorescence analysis with Arabidopsis cotyledons. The immunofluorescence signal of NAI2 was detected in the ER body, but not in the ER network. These data demonstrate that NAI2 is an ER body component that accumulates specifically in the ER body. We examined the effect of *NAI2* RNAi (*NAI2*-RNAi Arabidopsis lines) on ER body density and shape. ER bodies in *NAI2*-RNAi lines were fewer in number and were longer than wild-type ER bodies. These results indicate that NAI2 regulates the number and shape of ER bodies in Arabidopsis.

The *NAI1* gene regulates the formation of the ER body and the expression of the *PYK10* gene, which encodes a major component of ER bodies.[11] The *nai1-1* mutant had lower *NAI2* mRNA levels when compared with wild-type plants, which suggests that NAI1 regulates the expression of the *NAI2* gene. In contrast, wild-type and *nai2* mutant plants did not differ in the

levels of expression of *PYK10* and *NAI1* mRNA, which indicates that *NAI2* gene deficiency does not affect the expression of *PYK10* and *NAI1*. We examined the levels of PYK10 in the *nai2* mutant using immunoblot analysis. High levels of PYK10 were detected in seven-day-old wild-type plants. In contrast, its levels were reduced in the *nai2* mutants. There was no change in the levels of BiP, which is an ER protein. These findings indicate that NAI2 deficiency in Arabidopsis specifically reduces the accumulation of PYK10, which is a major ER body protein. We examined the localization of GFP-PYK10 fusion protein in the *nai2* mutants. In wild-type Arabidopsis plants, GFP-PYK10 accumulated mainly in ER bodies. Surprisingly, GFP-PYK10 was uniformly distributed throughout the ER network in the *nai2* mutant. These results suggest that NAI2 deficiency triggers the diffusion of PYK10 throughout the ER network. Thus, NAI2 is responsible for the accumulation of PYK10 in ER bodies.

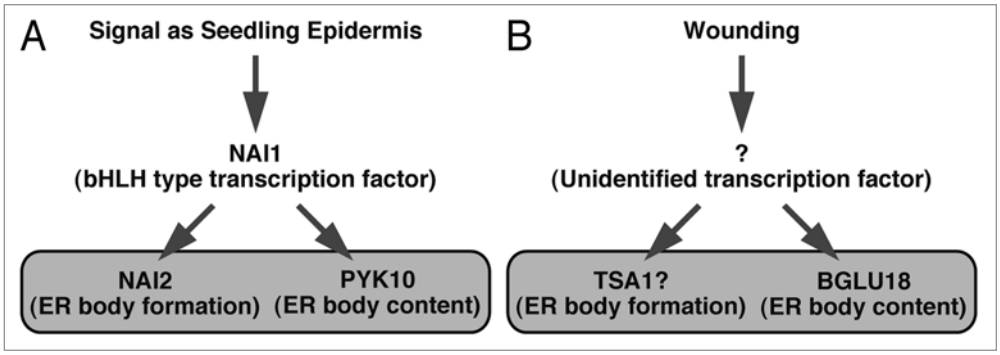

Figure 2. Model of ER body formation. (A) NAI1 regulates the expression of NAI2 and PYK10 in constitutive ER bodies of seedling epidermal cells. NAI2 is responsible for the formation of ER bodies and for the accumulation of PYK10. (B) Wound-induced ER bodies accumulate BGLU18, but not PYK10. A database analysis revealed that *TSA1*, which is a homolog of *NAI2*, is coexpressed with *BGLU18*. This suggests that TSA1 is involved in the formation of wound-induced ER bodies. The transcription factor involved in wound-induced ER bodies remains unknown.

Wounding Induces ER Bodies that Accumulate BGLU18

Wounding increases the number of ER bodies in cotyledons, which suggests that wounding induces the accumulation of other ER body components.[16] The BGLU family of Arabidopsis comprises 47 members.[17] Among these, eight highly homologous proteins (BGLU18/At1g52400, PYK10, BGLU19/At3g21370, BGLU20/At1g75940, BGLU21/At1g66270, BGLU22/At1g66280, BGLU24/At5g28510 and BGLU25/At3g03640) form a subfamily of proteins that carry both a signal peptide and a hypothetical ER retention signal. We analyzed the expression of these eight *BGLU* genes in wounded cotyledons and found that *BGLU18* is upregulated by ~five-fold in wounded cotyledons when compared with intact cotyledons, whereas the expression levels of *PYK10* remains constant.[16] The BGLU18 protein accumulates in wounded cotyledons, whereas only a small amount of BGLU18 protein is detected in intact cotyledons. In contrast, constant and similar levels of PYK10 protein are present in wounded and intact cotyledons.[16]

The Arabidopsis *nai1* mutant lacks ER bodies. However, ER bodies were induced in wounded cotyledons of the *nai1* mutant, although the ER bodies of the *nai1* mutant were tubular and not spindle shaped. ER bodies were mainly observed in cells surrounding the wounded site. In the *nai1* mutant, the expression of *BGLU18* was increased in wounded cotyledons, while *PYK10* was not expressed. The BGLU18 protein also accumulated in wounded cotyledons of *nai1* mutant plants. Immunogold electron microscopic analysis revealed that the BGLU18 protein accumulated in ER bodies in wounded cotyledons of the *nai1* mutant. These results suggest that BGLU18 is a main component of wound-induced ER bodies.

Conclusions and Perspectives

We identified NAI2 as an ER body protein that is responsible for the induction of ER bodies. In addition, we found that BGLU18 is a component of wound-induced ER bodies. These findings provide details on the formation of the ER body in Arabidopsis (**Fig. 2**). NAI1 regulates the expression of NAI2 and PYK10 and is responsible for ER body formation in seedling epidermis (**Fig. 2A**). As NAI2 deficiency reduced the accumulation of PYK10 and led to the diffusion of PYK10 throughout the ER network, NAI2 seems to be responsible for the accumulation and assembling of PYK10 in the ER body. In contrast, wound-induced ER bodies accumulate BGLU18, but not PYK10 (**Fig. 2B**). Therefore, it seems that Arabidopsis plants express at least two different components in two types of ER bodies. We searched the online database (ATTED-II; http://atted.jp/)[18] for genes that have an expression pattern that is similar to that of the *BGLU18* gene and found the *TSA1* gene, a *NAI2* homologue. This suggests that TSA1 is involved in the formation of wound-induced ER bodies (**Fig. 2B**). The transcription factor that is responsible for the induction of wound-induced ER bodies remains unknown. It is noteworthy that wound-induced ER bodies were present in the *nai1* mutant, but their shape was distorted.[12,17] This suggests that NAI1 is partly involved in the formation of wound-induced ER bodies.

The production of ER bodies is induced by wounding and by the wounding hormone jasmonate, which suggests that ER bodies may participate in pest/pathogen resistance.[5,7] Recently, Sherameti et al. reported that the *nai1* and *pyk10* mutants are hyperinfected by the root entophytic fungus *Piriformospora indica* and show reduced growth.[19] This suggests that the ER body may play a role in plant resistance against fungal infection. To elucidate the functions of the ER body, it is necessary to identify the substrates of PYK10 and of BGLU18.

As *NAI2* homologous genes are restricted in Brassicales, their ultimate function may be the promotion of pest/pathogen resistance in these plants. Further attempts to identify *NAI2* homologous genes in other Brassicales plants and neighboring orders may reveal how and why Brassicales plants developed *NAI2* homologous genes together with ER bodies. This, in turn, may provide new insights into the specific evolutionary genetic pathways that result in the appearance of new cellular structures.

Acknowledgements

This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (no. 21770057 to K.Y., nos. 16085203 and 17107002 to I.H.-N. and no. 16085209 to M.N.), and by Grants-in-Aid for the JSPS Fellows (no. 18003246 to A.J.N.) and from The Graduate University for Advanced Studies (Sokendai).

References

1. Galili G. ER-derived compartments are formed by highly regulated process and have special functions in plants. Plant Physiol 2004; 136:3411-3.
2. Hara-Nishimura I, Matsushima R, Shimada T, Nishimura M. Diversity and formation of endoplasmic reticulum-derived compartments in plants. Are these compartments specific to plant cells? Plant Physiol 2004; 136:3435-9.
3. Matsushima R, Hayashi Y, Yamada K, Shimada T, Nishimura M, Hara-Nishimura I. The ER body, a novel endoplasmic reticulum-derived structure in Arabidopsis. Plant Cell Physiol 2003; 44:661-6.
4. Hayashi Y, Yamada K, Shimada T, Matsushima R, Nishizawa N, Nishimura M, et al. A Proteinase-storing body that prepares for cell death or stresses in the epidermal cells of Arabidopsis. Plant Cell Physiol 2001; 42:894-9.
5. Matsushima R, Hayashi Y, Shimada T, Nishimura M, Hara-Nishimura I. An endoplasmic reticulum-derived structure that is induced under stress conditions in Arabidopsis. Plant Physiol 2002; 130:1807-14.
6. Matsushima R, Kondo M, Nishimura M, Hara-Nishimura I. A novel ER-derived compartment, the ER body, selectively accumulates beta-glucosidase with an ER-retention signal in Arabidopsis. Plant J 2003; 33:493-502.
7. Hara-Nishimura I, Matsushima R. A wound-inducible organelle derived from endoplasmic reticulum: a plant strategy against environmental stress? Curr Opin Plant Biol 2003; 6:583-8.
8. Behnke H-D, Eschlbeck G. Dilated cisternae in Capparales: an attempt towards the characterization of aspecific endoplasmic reticulum. Protoplasma 1978; 97:351-63.
9. Iversen T-H. Cytochemical localization of myrosinase (beta-thioglucosidase) in root tips of *Sinapis alba*. Protoplasma 1970; 71:451-66.
10. Iversen T-H. The morphology, occurrence, and distribution of dilated cisternae of the endoplasmic reticulum in tissues of plants of the Cruciferae. Protoplasma 1970; 71:467-77.
11. Matsushima R, Fukao Y, Nishimura M, Hara-Nishimura I. *NAI1* gene encodes a basic-helix-loop-helix-type putative transcription factor that regulates the formation of an endoplasmic reticulum-derived structure, the ER body. Plant Cell 2004; 16:1536-49.
12. Nagano AJ, Fukao Y, Fujiwara M, Nishimura M, Hara-Nishimura I. Antagonistic jacalin-related lectins regulate the size of ER body-type beta-glucosidase complexs in *Arabidopsis thaliana*. Plant Cell Physiol 2008; 49:969-80.
13. Nagano AJ, Matsushima R, Hara-Nishimura I. Activation of an ER-body-localized beta-glucosidase via a cytosolic binding partner in damaged tissues of *Arabidopsis thaliana*. Plant Cell Physiol 2005; 46:1140-8.
14. Yamada K, Nagano AJ, Nishina M, Hara-Nishimura I, Nishimura M. NAI2 is an endoplasmic reticulum body component that enables ER body formation in *Arabidopsis thaliana*. Plant Cell 2008; 20:2529-40.
15. Suzuki T, Nakajima S, Morikami A, Nakamura K. An Arabidopsis protein with a novel calcium-binding repeat sequence interacts with TONSOKU/MGOUN3/BRUSHY1 involved in meristem maintenance. Plant Cell Physiol 2005; 46:1452-61.
16. Ogasawara K, Yamada K, Christeller JT, Kondo M, Hatsugai N, Hara-Nishimura I, et al. Constitutive and inducible ER bodies of *Arabidopsis thaliana* accumulate distinct beta-glucosidases. Plant Cell Physiol 2009; 50:480-8.
17. Xu Z, Escamilla-Treviño LL, Zeng L, Lalgondar M, Bevan DR, Winkel BSJ, et al. Functional genomic analysis of *Arabidopsis thaliana* glycoside hydrolase family 1. Plant Mol Biol 2004; 55:343-67.
18. Obayashi T, Kinoshita K, Nakai K, Shibaoka M, Hayashi S, Saeki M, et al. ATTED-II: a database of co-expressed genes and cis elements for identifying co-regulated gene groups in Arabidopsis. Nucl Acids Res 2007; 35:863-9.
19. Sherameti I, Venus Y, Drzewiecki C, Tripathi S, Dan VM, Nitz I, et al. PYK10, a beta-glucosidase located in the endoplasmic reticulum, is crucial for the benefitical interaction between *Arabidopsis thaliana* and endophytic fungus *Piriformospora indica*. Plant J 2008; 54:428-39.